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Studies of Vaccinia Hemagglutinin Obtained from Various Vaccinia Infected Tissues. Density Gradient Centrifugation and Electron Microscopy.* (29934)

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(Introduced by T. Francis, Jr.)
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Because of its role as a by-product of viral infection and its properties as a lipoprotein, vaccinial hemagglutinin (VHA) has attracted the interest of several investigators. VHA was first described by Nagler(1) and subsequently the dissociation of VHA from infective vaccinia virus was demonstrated by adsorption onto red cells(2), centrifugation(2, 3), or by column chromatography(4). It has been characterized as a lipoprotein(3,5) and its density estimated as 1.1 and its size as 65 mμ(3). Stone(5) noted that lipids extracted from non-infected tissues would also agglutinate chicken red cells sensitive to VHA. Since only red cells from certain chickens were affected, she termed such cells "lipid sensitive" and the general phenomenon "lipid hemagglutination."

VHA can, however, be differentiated serologically from normal tissue lipid hemagglutination using specific immune serum(3,5). VHA is inhibited by specific immune serum; normal tissue lipid hemagglutination is inhibited by certain serum or tissue components which occur in some pathologic conditions(6,7,8). Lecithinase or snake venom phospholipase will destroy the activity of VHA and normal tissue lipid. The hemagglutinating activity of both is relatively heat stable at least in crude preparations.

Gillen *et al*(9) reported the occurrence of 2 viral specific particles, both with hemagglutinating activity and separable by filtration or high speed centrifugation. They did not differ serologically but were unlike in that the filterable particle which was non-sedimentable at 17,000 RPM was destroyed after a short incubation at 56°C. Briody(10) suggested that these differences were attributable to the degree of aggregation in each preparation and not to some inherent difference.

Youngner and Rubinstein(11,12) have described 2 hemagglutinins in infected chorioallantoic membrane separable by an ultracentrifugal flotation technique. The first or "viral" hemagglutinin is sedimented at 30,000 RPM and retains its serologic specificity. The second or "soluble" hemagglutinin floats to the surface at this speed (in a medium of 1.063 density) and serologically resembles the lipids of non-infected tissue. The activity of this soluble hemagglutinin is enhanced by dialysis or treatment with trypsin and is heat labile unlike viral hemagglutinin which is unaffected by any of these treatments. It would appear that these 2 hemagglutinins are the same as those originally reported by Stone and Burnet(2,5).

The only published electron micrographs (13,14) purporting to show vaccinial hemag-

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glutinin were made from crude preparations which still contained large amounts of virus. In view of the questions still unanswered regarding the structure and origin of vaccinial hemagglutinin the present study was undertaken to obtain purified and concentrated vaccinial hemagglutinin for morphologic, physical and serologic characterization.

Methods. 1) The Virus. The strain of vaccinia virus used in these studies originated from commercial lymph vaccine and is routinely passaged on chorioallantoic membrane (CAM) of eleven-day-old embryonate eggs. Material extracted from CAM was used to infect cultures of HeLa and mouse fibroblast cells and additional CAM.

2) Preparation of Vaccinial Hemagglutinin. Continuous passage HeLa cell cultures in the growth phase were rinsed free of media and infected with virus diluted in maintenance medium (Scherer's solution plus 10% inactivated horse serum) to a concentration of ca. 5×10^6 pock-forming units per ml. The virus was allowed to remain in contact with the cell sheet for 4 hours at 37°C; unabsorbed virus was removed by rinsing the cell sheet twice with balanced salt solution and then fresh maintenance medium added. Thirty to thirty-six hours after the initial infection, cells still adherent to the glass were scraped into the overlying fluid and the suspension centrifuged at 1000 RPM for 15 minutes to separate cells and large aggregates of debris from the fluid.

A primary passage of mouse embryo fibroblasts (MF) which had formed a confluent sheet were similarly infected and allowed to incubate an additional 36-40 hours before harvesting the cell and fluid phases. A preliminary study indicated that VHA appeared 6 to 8 hours later in infected mouse fibroblast cultures than in HeLa cells.

The sedimented cells were resuspended in a volume of 0.1 M citrate in saline equal to 1/10th that of the original suspension (*i.e.*, if the total volume of fluid and cells was 240 ml, the cells were resuspended in 24 ml of citrate saline) and disrupted by sonic oscillation. After another low speed centrifugation to remove debris the resulting opalescent suspension served as crude VHA of tissue culture origin.

The chorioallantoic membrane (CAM) of 11-day-old embryonate eggs was infected with 0.2 ml of 10^{-3} dilution of stock virus. Membranes with well developed pocks were harvested from living embryos 48 hours after inoculation. They were rinsed, weighed and made up to 20% suspension (w/v) in McIlvaine's buffer and blended in a mechanical homogenizer. Gross debris was removed by centrifugation at 1000 RPM for 15 minutes.

The crude hemagglutinin preparation (of HeLa, MF or CAM origin) was centrifuged at 10,000 RPM for one hour in the #30 rotor of the Spinco Model L ultracentrifuge. The supernate was centrifuged twice more at 10,000 RPM to remove as much virus as possible. The supernate from the third 10,000 centrifugation was then centrifuged for one hour at 40,000 RPM in the #40 Spinco rotor. The last centrifugation served to sediment the hemagglutinin which was resuspended in a volume of buffer equal to the weight of tissue initially used. The suspended sediment was further dispersed by sonication and served as starting material for density gradient centrifugation. The hemagglutinin titer of these preparations ranged from 1:160 to 1:2560 from HeLa cell cultures, 1:640 from mouse fibroblast cell cultures and 1:640 to 1:5120 from egg chorioallantoic membrane.

3) Density Gradient Centrifugation. Using a double chambered mixing and dispensing device(15), linear gradients of cesium chloride or sucrose were prepared in 4.1 to 4.6 ml amounts directly into celluloid tubes for use in the Spinco SW 39 rotor. A volume of hemagglutinin varying from 0.4 to 1.0 ml was layered on top and then centrifuged at 37,000 RPM for 15-18 hours. After centrifugation, the presence of any visible bands was marked on the tube exterior and the contents collected in dropwise fractions from a hole in the bottom of the tube. If the same material was centrifuged in all 3 tubes of the rotor, the fractions of each were collected in a single series of collecting tubes; this was necessary when the fractions were dialyzed and used in a second density gradient centrifugation. After measuring the volume of each fraction its buoyant density was determined by weight, using a volumetric microliter pipette (100 λ). The hemagglutinin titer of

TABLE I. Serologic Specificity of VHA Obtained by Density Gradient Centrifugation.

Preparation	Hemagglutination titer after 2 hr incubation with: Normal rabbit serum (1:10)	Immune rabbit serum (1:10)
Crude VHA (CAM) 40,000 RPM	1:2560	<10
1st DG centrifugation:		
Fraction A* D = 1.120	1:256	<10
" B D = 1.110	1:128	<10
" C D = 1.063	1:128	<10
2nd DG centrifugation:		
Fraction A D = 1.115	1:80	<10
" B D = 1.104	1:32	<10
" C D = 1.063	1:64	<10

* Same fractions shown in Fig. 4.

each fraction was determined as described below and the results expressed as a per cent of total activity *recovered* in all fractions.

In those experiments where fractions were recentrifuged in a second density gradient, they were first dialyzed to remove the cesium chloride or sucrose in which they were contained. The dialyzed sample was redispersed by sonication just before centrifugation.

4) Serologic Procedures. Hemagglutinating activity of the preparations was determined using a microtechnique described by Sever(16) with equipment available commercially. The test system described by Kempe(17) for VHA was adapted by reducing the volume used in the test ten-fold. A more consistent cell pattern in the negative (cell) controls was observed when a small amount of protein was present. Inactivated normal rabbit serum was added to saline to give a final concentration of 1:100 in the test and used to dilute the lipid sensitive chicken red cells added as a 0.5% suspension. This concentration of normal serum would inhibit hemagglutination by any tissue lipids also present as contaminants. However, each of the hemagglutinin preparations was tested at several stages of preparation for specificity; *i.e.*, tested for inhibition by specific vaccinial immune serum of human or rabbit origin but not by high concentrations of normal serum.

Results. Although the cycles of preparative centrifugation should eliminate those tissue lipids or lipoproteins of low density, the serologic specificity of selected fractions as well as that of the original starting material was determined by hemagglutination inhibition. As shown in Table I, hemagglutination was inhibited by rabbit immune serum but was unaffected by the same dilution of normal rabbit serum. In addition, non-infected HeLa cell cultures were carried through the same procedure outlined for virus infected cultures. None of the density gradient fractions obtained from non-infected cultures had hemagglutinating activity for lipid sensitive chicken red cells even when tested in a serum free system. Thus, in each instance hemagglutinin was viral specific and all were serologically related.

However, preliminary studies with VHA from HeLa and CAM prepared in density gradients suggested that they differed significantly in buoyant density. Further experiments employing MF as an additional host system confirmed this observation (Fig. 1). VHA prepared from infected HeLa cells was most dense, that from CAM had an intermediate density and from MF, least dense. Density varied slightly depending on whether the gradient was prepared from cesium chloride or sucrose; however, the type of host cell system infected was the major factor in determining density. The effect of the composition of the gradient on the range of densities observed in various experiments is shown in Table II. When VHA from HeLa and MF were recentrifuged separately and

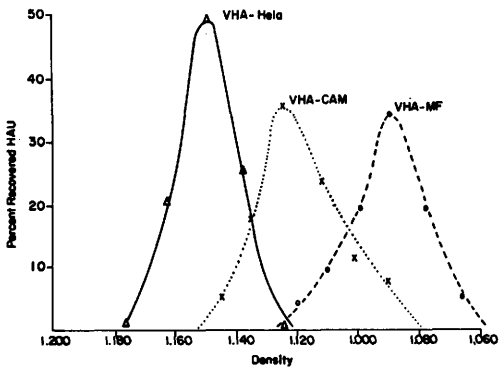


FIG. 1. Distribution of VHA from various tissues by density and percent HAU recovered (composite of separate experiments using cesium chloride and sucrose gradients).

TABLE II. Effect of Density Medium on Observed Density of Vaccinial Hemagglutinin.

Density medium	Tissue source	Observed density range
Cesium chloride	HeLa	1.175–1.168 (3)*
	CAM	1.121–1.098 (2)
Sucrose	HeLa	1.147–1.130 (3)
	CAM	1.113–1.065 (3)

CAM = chorioallantoic membrane.
* ( ) = No. of experiments.

in combination each was reisolated at the same density as that originally observed and the combination of both showed 2 peaks of activity, each of which corresponded in position to that of the separate preparations (Fig. 2).

Using more highly concentrated starting material, larger quantities of VHA were obtained with high titers (1:2500-1:5000) from HeLa and CAM. When subjected to density gradient centrifugation, some heterogeneity

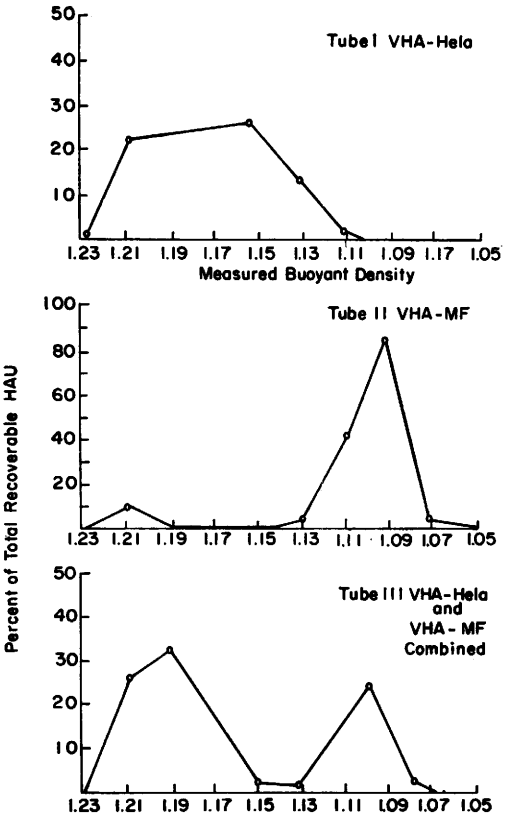


FIG. 2. Density gradient centrifugation of vaccinial hemagglutinin from 2 different tissues alone and in combination.

of the preparations with regard to minor components was revealed with a resulting loss of resolution and some overlapping of the densities of VHA from different host systems. If single fractions of the initial gradient were dialyzed and recentrifuged, they yielded single relatively sharp peaks of good resolution at the same density at which they had been isolated in the first centrifugation. Four preparations, 2 from HeLa and 2 from CAM, were examined in this way and the results of each resembled that shown in Fig. 3. Therefore, the results of the initial cen-

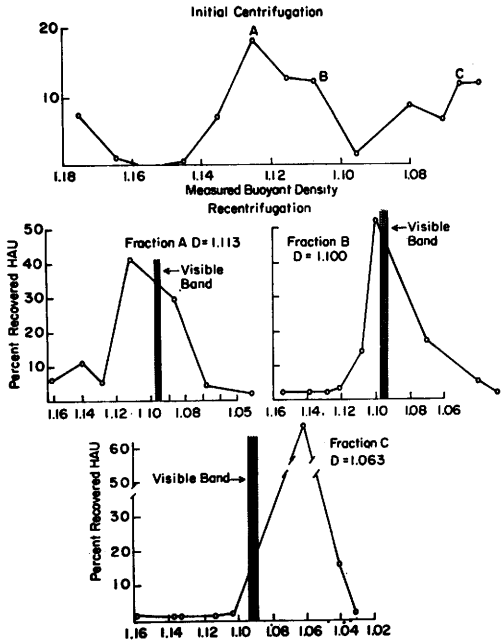


FIG. 3. Density gradient centrifugation of VHA from chorioallantoic membrane.

trifugation are indicative of a marked degree of heterogeneity which if due to poly-dispersion was not further resolved by a second density gradient centrifugation. Briody(10) had previously commented on the poly-disperse nature of VHA in relation to the report by Gillen *et al*(9) reporting 2 varieties of hemagglutinin, both serologically specific. When high titered, concentrated preparations are used, aggregates of several particles are more likely to form so that a mixture of trimers, dimers and single units is being separated. Such aggregates would differ in sedimentation velocity to some degree. Although

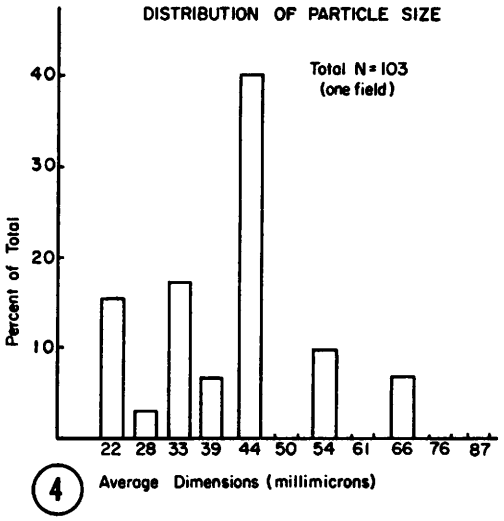
VHA is stable to heating, on storage a 2- to 4-fold drop in titer is often encountered; resonication will restore the titer to its original level.

The preparations described here were centrifuged for 18-20 hours in a preformed density gradient. To be certain that this time interval was adequate to obtain equilibrium, additional gradients were centrifuged for 48 and 72 hours and then harvested and assayed as usual. The poly-dispersion previously observed with high titered preparations was unaltered by prolonged centrifugation and cannot, therefore, be attributed to inadequate time of centrifugation.

Electron micrographs of VHA (HeLa) indicated that the particle size was 44 mμ in shadowed preparations (Fig. 4), Chu(3) estimated the particle size as 65 mμ from sedimentation studies and more recently McCrea (18) reported a value of 46 mμ, based on inactivation by ionizing radiation. The material when examined in an electron microscope was not homogenous, for several classes of particle size were apparent. The most frequent particle had an average dimension (based on length and width) of 44 mμ and accounted for 40% of the total number counted and measured (Fig. 4).

Examination of shadowed grids made from another sample of the same preparation after absorption with ghosts of lipid sensitive chicken erythrocytes confirmed this observation. The total number of particles was considerably reduced and it was necessary to examine 3 fields to count a comparable number of particles. Although not all 44 mμ particles have been absorbed (Fig. 5), the particle distribution is quite different so that particles of less than 30 mμ predominate. The observation of a progression of classes of particle sizes (*e.g.*, 22 mμ, 33 mμ, 44 mμ) suggests a repeating subunit and that the larger particles may be aggregates. However, since the smaller particles are not adsorbed to the red cell they cannot be related as subunits to the hemagglutinating particle with certainty.

Micrographs of red cell ghosts with adsorbed VHA showed that the entire surface of the cell was saturated with particles (Fig. 6). The distribution of particle sizes seems at odds with the unabsorbed preparation since the predominant particle has an average dimension of 54 mμ with a number of larger particles not seen in the unabsorbed preparation. On close inspection, an irregularity in dimensions was seen frequently in contrast to the earlier picture and occasionally obvious dimers or trimers may be discerned. The alteration in size from 44 mμ to 55 mμ may be due to the binding between the par-



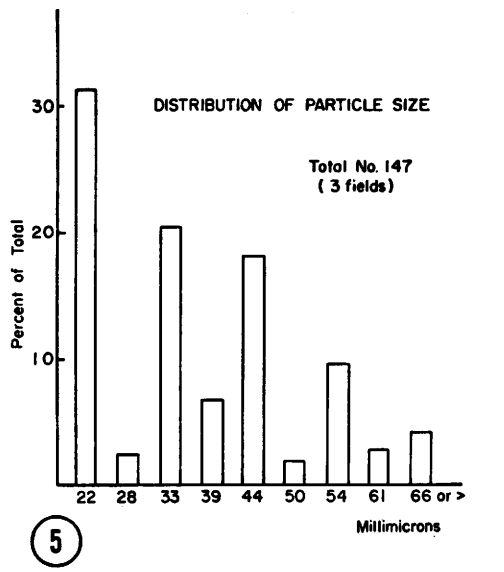


FIG. 4. Particle size distribution of vaccinial hemagglutinin (shadowed preparation).

FIG. 5. Particle size distribution of vaccinial hemagglutinin after absorption onto red cell.

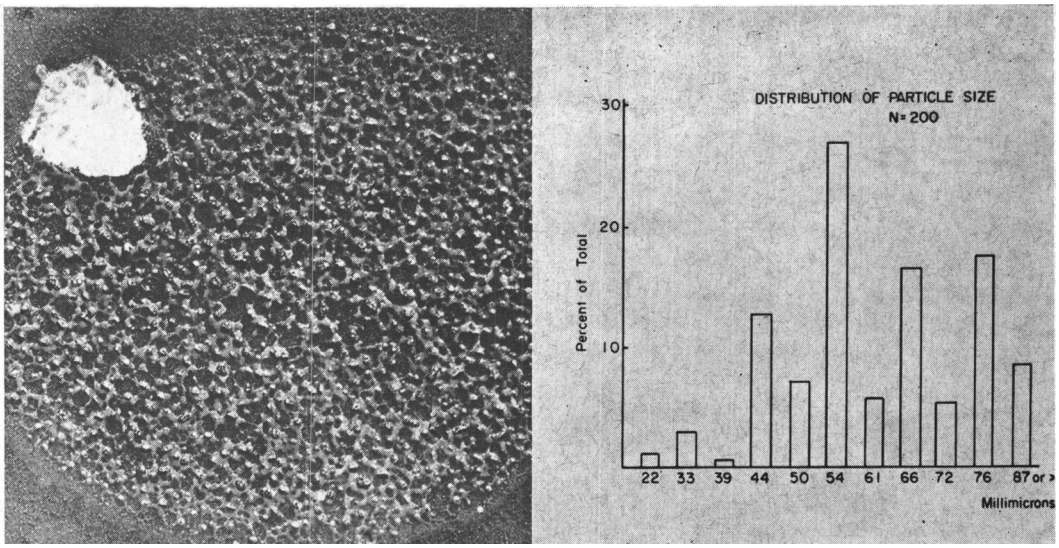


FIG. 6. Electron micrograph (× 27,600) and particle size distribution of vaccinial hemagglutinin absorbed onto red cell surface.

ticle and red cell surface, a remarkably firm one. Chu(3) has reported on the difficulty with which VHA can be eluted from the red cell surface even under extremes of pH, temperature or isotonicity. It seems likely that the particle dimensions may be altered by such binding so that the adsorbed particle is distorted or flattened, thus increasing the average dimension, based on length and width. Enlargements of VHA (Fig. 7A) show an absence of clearly defined boundaries and 2 particles (arrows) suggest that the particle has a strand-like substructure; a similar substructure is also seen (Fig. 7B) in particles adsorbed onto an erythrocyte ghost. Based on these photographs, VHA appears to be constructed by the coiling or folding of a strand-like macromolecule into a semispherical shape of somewhat variable dimension and density.

Discussion. The variation in density which is host determined supports the concept originally advanced by Chu(3) that VHA is a cellular by-product of the virus infection, and, although not a viral component, arises in response to viral-induced cellular damage. Knight(19) and Frommhagen *et al*(20) proposed that normal host cell components are incorporated into the influenza viral particle on the basis of serologic evidence of cross reactivity and from chemical analyses of the lipid components. More recently, Kates (21) has reviewed the evidence and also offered new data which support the original suggestion of Knight. Stenback and Durand (22), using density gradient centrifugation have demonstrated that the host tissue of origin may in part at least contribute to the density of purified Newcastle Disease virus. Gaush and Youngner(23) have investigated the lipids of vaccinia infected CAM and have observed a marked alteration after infection as reflected by the change in lipid composition of vaccinia infected CAM. The percentage increase in triglycerides and phosphatidyl inositol of infected CAM and insoluble hemagglutinin were striking.

This increase in total lipid together with the increases in protein observed in vaccinial infected HeLa cells and CAM (Ackermann and Loh(24), Gaush(25)), may result in a milieu favorable for the formation of an abnormal lipoprotein, antigenically foreign to the host, which is a hybrid of cellular and viral induced components. Loh and Riggs (26) have shown that VHA first appears well after the initial production of infectious virus. Oda(27), studying the effect of specific immune serum on formation of vaccinial hemagglutinin in the infected cell, has observed that addition of immune serum results in disappearance of VHA without affecting the pro-

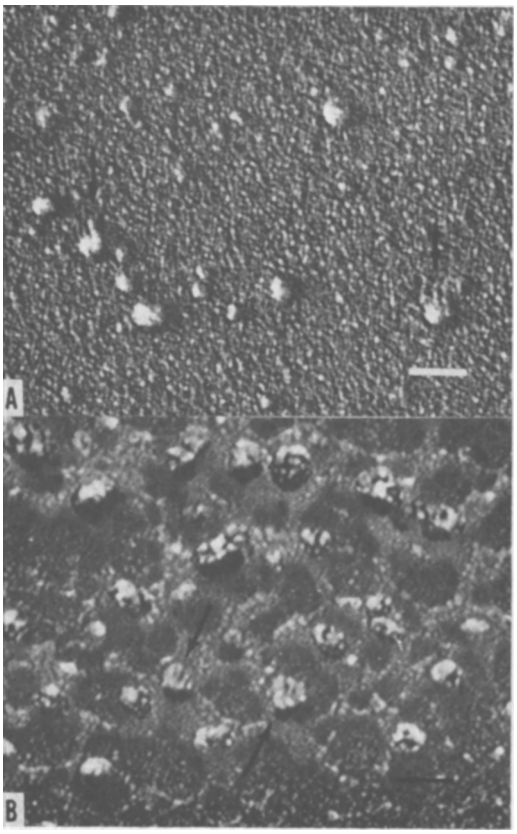


FIG. 7. Enlargements of electron micrograph of vaccinial hemagglutinin. A: Shadowed grid 9,000 ×; bar = 100 μm. B: Adsorbed to red cell surface 90,000 ×; bar = 100 μm.

duction of infective virus. Following removal of immune serum VHA is formed and is released at the same rate seen in cells not exposed to immune serum after infection. He concludes that formation of VHA occurs at or near the cell surface from sub-units resulting in part from the viral multiplication which is taking place more interiorly within the cell and is unaffected by the external presence of immune serum.

The spontaneous formation of such lipoprotein complexes might well be a process with inherent variability giving rise to the heterogeneous character of the isolated VHA; such heterogeneity would not be an artifact of extraction. Within a restricted range some degree of randomness may result in the size of the particle which is formed by the coiling of the strand of lipoprotein. The classes of phospholipid or lipid contributed by the specific host cell which combine with available protein would result in the observed differences in density. The heterogeneity of concentrated, high-titered preparations not resolvable by further density gradient centrifugation argues for the presence of several classes of particles all with similar hemagglutinating activity and serologic specificity.

The electron micrographs are consistent with this hypothesis; the alteration of dimensions when the particle is adsorbed to the red cell surface and the coiled particles seen (Fig. 7) support the concept that VHA possess a certain plasticity which may account for its lack of a rigid well defined structure.

Summary. Serologically specific vaccinial hemagglutinin (VHA) was obtained from vaccinia infected HeLa cells, chorioallantoic membranes of embryonated eggs and mouse fibroblast cell cultures. When concentrated and partially purified preparations were examined by density gradient centrifugation, the preparations varied in density according to the tissue of origin. The host-associated differences in peak density were accompanied by a marked degree of poly-dispersion not resolvable by a second density gradient centrifugation or by prolonged centrifugation. Electron micrographs showed that the particle size was in the range of 45-54 mμ. Substructure was detected in some particles which suggested the coiling or folding of a strand.

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Further Evidence of a High Degree of Genetic Homology Between *H. influenzae* and *H. aegyptius.** (29935)

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DNA-mediated interspecific transformations have been reported for a number of bacterial "species" or groups(1) and are interpreted as evidence of some degree of genetic homology among the groups which exchange genetic material (DNA). Among "species" of Hemophilus, the heterospecific to homospecific transformation ratio is low for *H. influenzae* populations exposed to *H. parainfluenzae* or *H. suis* DNA and for *H. parainfluenzae* cells exposed to *H. influenzae* DNA (2). On the other hand, this ratio may approach unity at times when *H. influenzae* and *H. aegyptius* populations are considered(3); a high degree of genetic homology for these 2 "species" is suggested. This conclusion was based on data from experiments in which streptomycin (S) resistance was used as a genetic marker. The present study extends this analysis of interspecific transformation in *H. influenzae* and *H. aegyptius* to include resistance to novobiocin (NV) as a genetic marker. NV resistance is linked to S resistance in *H. influenzae*(4). The data presented show a high heterospecific to homospecific transformation ratio for these markers in members of the 2 "species" and indicate that NV resistance is linked to S resistance in *H. aegyptius* also. The data also show a "species" difference in sensitivity to NV which is reflected in the level of NV resistance conferred by the heterologous species NV resistance transforming factor.

Materials and methods. Recipient populations. Strains R*b* and R*d* *H. influenzae* and 15 and 181*a* *H. aegyptius*(3). We are indebted to Dr. Margaret Pittman for *H. aegyptius* strains.

DNA donor populations. 1) *H. influenzae* R*d* S 1000 NV 5; nonencapsulated variant derived from type *d*; resistant to 1000 μg S per ml and 5 μg NV† per ml. A NV resistant population was selected first by seeding 5×10^7 sensitive cells in pour plate preparations of Levinthal agar containing 2 μg NV per ml. A single colony which formed in this environment within 48 hours incubation was

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